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Cortistatin promotes and negatively correlates with slow-wave sleep

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Abstract

Sleep need is characterized by the level of slow-wave activity (SWA) and increases with time spent awake. The molecular nature of this sleep homeostatic process is practically unknown. Here, we show that intracerebroventricular administration of the neuropeptide, cortistatin (CST-14), enhances EEG synchronization by selectively promoting deep slow-wave sleep (SWS) during both the light and dark period in rats. CST-14 also increases the level of slow-wave activity (SWA) within deep SWS during the first two hours following CST-14 administration. Steady-state levels of preprocortistatin mRNA oscillate during the light : dark cycle and are four-fold higher upon total 24-h sleep deprivation, returning progressively to normal levels after eight hours of sleep recovery. Preprocortistatin mRNA is expressed upon sleep deprivation in a particular subset of cortical interneurons that colocalize with *c-fos*. In contrast, the number of CST-positive cells coexpressing pERK1/2 decreases under sleep deprivation. The capacity of CST-14 to increase SWA, together with preprocortistatin's inverse correlation with time spent in SWS, suggests a potential role in sleep homeostatic processes.

Introduction

Sleep regulation involves two main regulatory processes, i.e. a circadian and a homeostatic component (Borbely & Wirz-Justice, 1982; Borbely & Achermann, 1999). The circadian process strongly regulates the distribution of sleep over the 24-h cycle. The homeostatic process tracks sleep need and is a common phenomenon in invertebrates (Shaw *et al*., 2000; Hendricks *et al*., 2001) and mammals (Franken *et al*., 2001). Sleep homeostasis implies sleep need increases as a function of sustained wakefulness resulting in compensatory changes in sleep duration, and especially in sleep depth during subsequent sleep following prolonged wakefulness. It has long been shown in humans that deep nonrapid-eye-movement (NREM) sleep, i.e. slow-wave sleep, is homeostatically regulated (Knoblauch *et al*., 2002). Time spent in deep sleep (defined by the presence of high amounts of high amplitude slow waves, i.e. NREM stage 3 and 4 in humans and NREM stage 2 in rats) as well as delta power of the EEG (0.5–4 Hz) are indicators of slow-wave activity and are robust physiological markers of sleep need. Sleep homeostasis has been associated with synaptic plasticity (Huber *et al*., 2004; Huber *et al*., 2006) and the role of slow-wave sleep oscillations in consolidating memory traces acquired during wakefulness has been explored in animals and humans. It has been recently suggested that synaptic potentiation is associated with the homeostatic regulation of slow-wave activity (SWA) and that SWA is linked with synaptic downscaling, which is related to the beneficial effect of sleep on cortical functioning and performance (Tononi & Cirelli, 2006). Besides adenosine (Porkka-Heiskanen *et al*., 2002) and pro-inflammatory cytokines (Obal & Krueger, 2003), few molecules have been shown to correlate with sleep need.

Cortistatin (CST-14) is a neuropeptide that has strong structural similarity with somatostatin (SRIF-14), yet CST-14 and somatostatin are products of different genes (de Lecea *et al*., 1996). The similarity between the structures of CST-14 and SRIF-14 and their pharmacological properties might suggest that the *in vivo* role of CST-14 is similar to that of SRIF-14. However, this appears not to be the case as (i) CST-14 levels are not up-regulated in the somatostatin knock out mouse (Zeyda *et al*., 2001); (ii) preprocortistatin and preprosomatostatin genes respond to different signals (Calbet *et al*., 1999); (iii) the peptide precursors are expressed in partially overlapping but distinct populations of neurons (de Lecea *et al*., 1997); (iv) CST-14 exerts many markedly different physiological effects compared to SRIF-14

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(reviewed in Spier & de Lecea, 2000); (v) CST-14, but not somatostatin enhances the h-current, suggesting that they activate different intracellular signal transduction pathways (Schweitzer *et al*., 2003); and (vi) CST-14 binds to all somatostatin receptors and shows some selectivity for other receptors, including MrgX2 (Robas *et al*., 2003). Thus CST-14 does not appear to be a somatostatin back up, but a neuropeptide with distinct functions.

Here, we report the characterization of the sleep promoting effect of CST-14 and its negative correlation with time spent in NREM sleep. Cumulatively, our data suggest that CST-14 is an endogenous peptide that selectively affects EEG cortical synchronization.

Materials and methods

In vivo *experiments*

Studies in freely moving animals were performed on adult male Sprague–Dawley rats (Charles River Laboratory, Hollister, CA), weighing 260–325 g at the time of surgery, kept under control environmental conditions (12-h light : 12-h dark; 23 ± 1 °C, food and water *ad libitum*) and handled in agreement with the ethical rules for experimentation on laboratory animals in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Scripps Research Institutes IACUC. Animals were maintained in reverse (on 22:00 h, off 10:00 h) or on a normal 12-h light : 12-h dark cycle (on 06:00 h, off 18:00 h) for at least 14 days before surgery (i.e. 24 days before recording).

Sleep recordings and analysis

Animals were anaesthetized under halothane (1–2%) and implanted with a standard set of electrodes (two screw electrodes placed in the bone over the dura (2 mm lateral to the midline, 2 mm anterior to the bregma and 2 mm anterior to the lambda) and two wire electrodes inserted in the neck musculature) to record the electroencephalogram (EEG) and the electromyogram (EMG). A stainless steel guide cannula was also stereotaxically positioned 1 mm above the left lateral ventricle (Paxinos & Watson, 1986; from bregma: AP −0.9 mm, L 1.4 mm, V 2.6 mm under the brain) for drug administration. After a 10-day period of recovery and habituation to the recording conditions, synthetic CST-14 was dissolved in saline and a total volume of 4 μL was injected through a smaller cannula 2 mm longer than the guide tube over 10 min, after which the cannula was left in place for another 2 min. CST-14 was synthesized at Scripps' microchemistry facility and purified by HPLC (98% purity). Molecular mass and structure were confirmed by mass spectrometry. Peptide content was > 85%.

Polygraphic recordings and behavioural observation began immediately after the infusion of saline or CST-14 (random order, for 6 h; reverse cycle $n = 5$ rats; normal cycle $n = 12$ rats). Rats were injected 4 h after the onset of the light or dark cycles at 10:00 h in normal cycle and 14:00 h in reverse cycle. Recordings were also performed on the day after a drug treatment following a sham microinjection to verify that the sleep wakefulness amounts had returned to control values. Infusions of saline or CST-14 were given in random order using a Latin Square design.

The EEG and EMG signals were amplified simultaneously in a Grass Model 7D polygraph (Astro-Medical, Icn Product Group, West Warwick, RI, USA) in a frequency range of 0.25–35 Hz and sampled at 256 Hz. The EEG and EMG were displayed on a computer monitor and stored with a resolution of 128 Hz in a hard drive of a computer for the off-line analysis of the sleep-wake states and spectral analysis using software supplied by Kissei-Comtec (Irvine, CA, USA). The EEG and electromyogram were recorded over 6 h. The recording chambers contained a mini video camera for continuously observing animal behaviour during the recording sessions. Recordings were also performed the day after the infusion to verify that the sleep-wakefulness amounts had returned to control values.

Sleep recordings were visually scored every 15-s epoch into wakefulness (W), NREM and rapid-eye-movement (REM) sleep according to standard criteria (Neckelmann *et al*., 1994). NREM was subdivided into light (stage 1) and deep (stage 2) sleep based on the amount of high amplitude slow-wave activity (criteria defined for rats; Neckelmann & Ursin, 1993). Data were analysed for 2-, 4- and 6-h periods and expressed as mean ± SEM. The following variables were examined; time spent in wakefulness, SWS1, SWS2 or REM sleep; duration and number of REM sleep episodes; latency of SWS2 onset (interval between the time of injection and the first SWS2 episode of at least 15-s duration). The effects of each dose of CST-14 were analysed upon the parameters mentioned above.

EEG power spectra were computed for 4-s epochs by a Fast Fourier Transform (FFT) routine and 0.25-Hz bin values were averaged for each of four different vigilance states, and those above 25 Hz were omitted. For each animal the spectrogram values after administration of one dose of CST-14 were compared with their respective control values (saline injection). EEG spectrograms were expressed as a percentage of the EEG power for each frequency bin (0.25 Hz) over total EEG power (0.25–25 Hz). Because the analysis bin per bin revealed only significant changes in low frequencies, the delta frequency band was analysed further. The frequency band corresponding to slow-wave activity (SWA) was defined as pooled spectral values between 1.45 and 3.50 Hz (frequency window corresponding to the delta peak as observed from spectrum profiles in SWS2). Values for SWA following cortistatin injection are expressed as percentage of individual mean level of SWA over same period postinjection of saline (100%) (mean ± SEM 2-h period values). If artifacts occurred the epoch was discarded < 3% of recording time).

For a given dose, each animal was referred to its own baseline represented by the data obtained after saline injection. Statistical analyses for sleep parameters as well as spectral values were performed using repeated measures ANOVA, and in case of significance ($P < 0.05$), the *F*-test was followed by Fisher's least significant difference (LSD). The time course of the SWA was analysed specifically in each vigilance state for every 2-h period. The data from the two CST-14 doses were pooled because their distribution data showed an overlap between CST-14 100 ng and 1000 ng, without any statistical difference between the two doses. Thus, the cortistatin effect on delta power band in SWS2 was on/off, and a paired *t*-test was used in addition to the ANOVA.

Sleep deprivation

Total sleep deprivation was accomplished by gentle handling, as described previously (Franken *et al*., 1991; Toppila *et al*., 1997). Animals were constantly exposed to novel objects and changed to new cages four times. Several experimenters taking relays observed and manipulated the animals during the 24-h period. Rats ($n = 18$) were sleep deprived for 24 h starting at 06:00 h and were inspected continuously during the light period and during the dark period under red light. Rats assigned as controls ($n = 9$) for the total sleep deprivation group were undisturbed and maintained in the same housing conditions. Two groups of rats recovered from total sleep deprivation by allowing them to sleep for either four ($n = 3$) or eight ($n = 6$) hours before termination of the procedure. At the end of these procedures, rats were deeply anaesthetized with CO_2, brains were

removed and immediately processed for RNA extraction or animals were perfused intracardially with 4% paraformaldehyde in PBS, pH 7.4 for *in situ* hybridization and combined immunohistochemistry.

REM sleep deprivation

Rats ($n = 16$) were placed for 8 h, starting at 08:00 h, on platforms (control conditions 15 cm in diameter, 8-cm high; deprivation conditions 5.5 cm in diameter, 8-cm high) surrounded by water (2-cm deep) with access to food and water *ad libitum* at an ambient temperature (23 °C; Mendelson *et al*., 1974; Maudhuit *et al*., 1996). Animals were placed in the same conditions for very short periods of 10 min for habituation during the days before the procedure. At the end of this period, animals were returned to their home cage for recovery. These conditions of habituation to the large platform overcame the early REM deprivation effect. Based on our experience in our experimental conditions, NREM sleep, in particular SWS2, is highly preserved under the REM deprivation protocol, as well as REM sleep, which reaches baseline values in animals on the control platform (Lemasson *et al*., 2002).

Northern blot

Animals were killed by CO_2 inhalation. Cytoplasmic poly A^+ RNA from cortex of Sprague–Dawley rats was extracted as described by Schibler *et al*., (1980). Two micrograms of poly A^+ RNA, were run on agarose formaldehyde gels, transferred to nylon membranes and hybridized with a ^{32}P-labelled full-length rat preprocortistatin cDNA probe. The same membrane was hybridized with cyclophilin as a control probe for loading and RNA integrity (Danielson *et al*., 1988), and with *c-fos* (kindly provided by Dr James Morgan) as an estimate of neuronal activity. Film autoradiographs were scanned and the signal was quantified using ImageQuant software (Molecular Dynamics). Results were compared by one-way ANOVA followed by Fisher's LSD *posthoc* test $P < 0.05$.

Anatomical studies

We conducted *in situ* hybridization and combined immunohistochemistry essentially as described elsewhere (de Lecea *et al*., 1997). Three rats from each condition (control $n = 3$, sleep deprivation $n = 3$ and sleep deprivation and 8 h recovery $n = 3$) were utilized in this study. Animals were anesthetized with 5% halothane and perfused with 4% paraformaldehyde. Sense probes were used as negative controls (not shown). Thirty-five-micron thick free-floating sections were incubated with 1×10^6 cpm/mL of ^{35}S-labelled probe. All experimental conditions were run in parallel in each hybridization experiment, to allow semiquantitiative comparisons. For immunohistochemistry the following primary rabbit polyclonal antisera were used; antibodies to c-FOS (Oncogene sciences; 1 : 10 000) and ERK1/2 (New England Biolabs; 1 : 500). Immunostaining was detected using ABC-peroxidase with 0.5 mg/mL of diaminobenzidine and 0.01% H_2O_2 as peroxidase substrates.

Analysis of anatomical data

Sections were examined with a Zeiss microscope with 40× objective. The delimitation of regional and laminar boundaries was carried out according to Zilles (1985). For the quantitative analysis of labelled neurons, only sections processed in parallel under exactly the same conditions and displaying similar background levels (< 10 grains/100 μm^2) were considered. Cells and grains were counted by an experimenter blind to experimental conditions. As the autoradiographic background levels were less than six grains per cell, neuronal somata were considered positive when overlaid by 15 or more silver grains, although positive neurons normally displayed > 25 grains. Sections hybridized with a sense riboprobe never exceeded the background threshold. To determine the radial distribution of cortistatin mRNA positive cells, eight vertical strips (250-μm wide) covering the entire cortical thickness were harvested in the following areas; somatosensory (first parietal area, PAR1), motor (hindlimb-forelimb, HL-FL), visual (primary mono-binocular, occipital, OC1M-B) cortices and hippocampal CA1. The number of labelled cells in single layers was counted and their percentage relative to the remaining cortical laminae calculated. Densities of cortistatin-expressing cells were determined by counting positive cells in eight frames (650 × 440 μm) corresponding the cortical areas of three different animals.

For quantitative analysis of double labelled neurons, the number of immunoreactive cells displaying positive and negative hybridization in the somatosensory and visual/occipital cortices was counted in 6–15 sections from three animals for each neurochemical marker (c-FOS and pERK1/2).

Results

Cortistatin actively promotes deep slow-wave sleep

To determine whether the effects of CST-14 varied across the circadian cycle, we analysed the consequences of i.c.v. administration of CST-14 on sleep/wakefulness states in the dark and light periods. We infused two doses of peptide (100 ng and 1000 ng) in rats in the light period (injection time 10:00 h; lights on 06:00 h; lights off 18:00 h) and dark period (injection time 16:00 h; lights on 22:00 h; lights off 10:00 h) and recorded sleep for 6 h.

Administration of CST-14 produced a dose-dependent increase in the amounts of SWS2 in rats injected during the light (ANOVA, $F_{2,33} = 17.44$; $P < 0.001$; 0–6 h) and dark period (ANOVA, $F_{2,13} = 9.10$; $P < 0.01$; 0–6 h) (Fig. 1A). This effect was maximal for the first 4 h (Table 1) and was primarily accounted for by an increase in the duration (dark period $F_{2,13} = 8.20$, $P < 0.01$; light period, ns; 0–6 h) but not in the number of SWS2 episodes (Fig. 1B). SWS2 latency was not modified. The amounts of wakefulness decreased in rats injected under light ($F_{2,33} = 8.84$; $P < 0.01$; 0–6 h) and dark periods (Fig. 1A). A significant decrease in the amounts of SWS1 ($F_{2,33} = 6.78$; $P < 0.01$) was also observed after administration of the peptide in the light period. No changes in REM sleep amounts were noticed, whereas REM sleep latency was increased after injection of the higher dose of CST-14 in dark period (not shown, $F_{2,13} = 6.72$, $P < 0.01$). In order to determine whether CST-14 may increase the level of EEG cortical synchronization within SWS2, we analysed the spectral power profile and power time course of SWA. After administration of both doses of CST-14, the bin per bin analysis showed an increase in power density of several frequency bins that belong to the delta frequency band (Fig. 2A). Delta power was significantly increased in SWS2 during the first two hours of recording following CST-14 infusion (average 131.3% ± 13.3 compared with 100% saline 0–2 h; $P < 0.05$, $n = 5$; Table 2). This effect was not dependent on the dose of CST-14, but was statistically significant when both doses of CST-14 (100 ng and 1 μg) were compared with saline. Figure 2B illustrates the kinetics of the effect of CST-14 (100 ng) on EEG spectral profile and shows an effect on SWA mainly observed for the first hours following the injection. Besides frequency bins that belong to the delta frequency band, CST-14-induced NREM sleep showed a spectral power profile that was indistinguishable from spontaneous naturally occurring NREM sleep

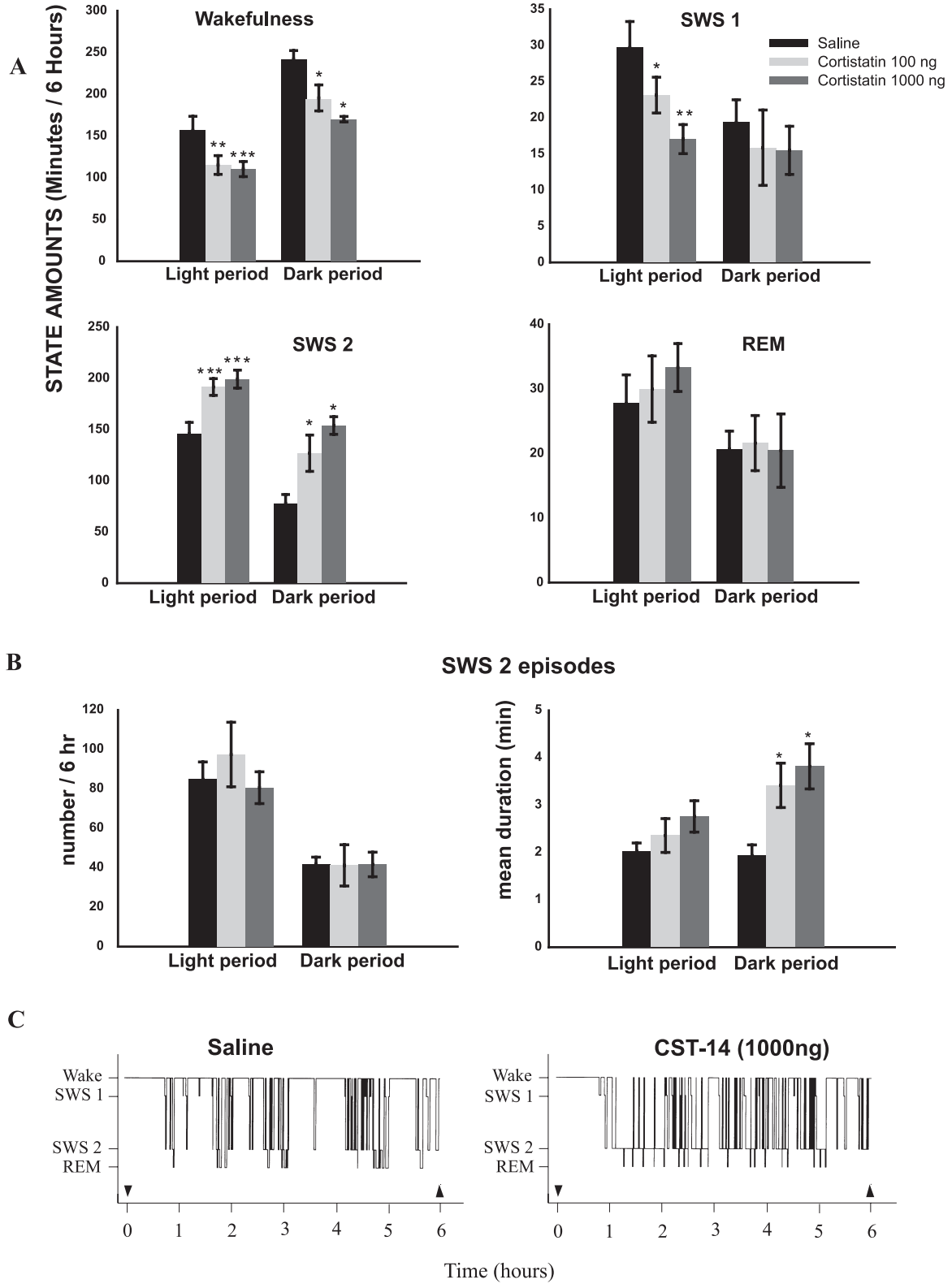

FIG. 1. Effects of CST-14 on wakefulness and sleep during 6 h following its i.c.v. infusion in both, light and dark periods. (A) Histograms showing amounts of vigilance states in minutes during 6 h following CST-14 i.c.v. administration. CST-14 induced a dose dependent increase in SWS2 (ANOVA, normal cycle $n = 12$, $P < 0.001$; reverse cycle $n = 5$, $P < 0.05$) amounts at the expense of wakefulness (normal and reverse cycle) and SWS1 (normal cycle). The asterisk indicates a significant difference as compared to vehicle injection (Fisher's *posthoc* test $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (B) Effect of CST-14 on SWS2 maintenance. The sleep-enhancing effect of CST-14 is accounted for by a dose dependent increase in the duration of sleep episodes (ANOVA followed by Fisher's *posthoc* test $^*P < 0.05$) for normal and reverse cycle. No significant changes in the number of episodes were observed. (C) Representative hypnogram of a rat injected with 1 μg of CST-14 into the lateral ventricle during the dark period. Note a pronounced enhancement of SWS2 and a concomitant decrease in wakefulness.

TABLE 1. Time course of the effect of CST-14 on vigilance states

Vigilance state and CST-14 test	Normal cycle (state duration in minutes)†			Reverse cycle (state duration in minutes)†		
	0–2 h	2–4 h	4–6 h	0–2 h	2–4 h	4–6 h
W						
Saline	48.6 ± 4.8	48.5 ± 7.4	59.5 ± 5.9	88.3 ± 3.5	79.3 ± 4.8	74.1 ± 5.7
100 ng	34.9 ± 3.0**	33.0 ± 3.4**	47.5 ± 6.8	72.0 ± 8.5	50.7 ± 10.4*	72.9 ± 11.3
1000 ng	35.8 ± 4.0**	31.0 ± 3.6***	43.7 ± 4.5	83.0 ± 12.7	14.5 ± 9.8*	51.2 ± 7.8
Saline	9.6 ± 1.3	10.1 ± 1.6	10.2 ± 1.1	6.2 ± 1.5	6.1 ± 0.1	7.1 ± 1.2
SWS1						
100 ng	7.6 ± 0.8*	7.9 ± 1.2	7.7 ± 1.1*	5.1 ± 1.6	5.1 ± 2.5	4.9 ± 1.6
1000 ng	4.6 ± 0.6***	5.7 ± 0.8	6.7 ± 0.8*	4.8 ± 1.3	5.2 ± 1.7	6.2 ± 1.7
Saline	52.9 ± 3.5	51.8 ± 5.2	41.3 ± 3.8	22.4 ± 3.1	25.7 ± 4.2	28.1 ± 3.7
SWS2						
100 ng	68.4 ± 2.8**	67.6 ± 2.7***	55.0 ± 5.1*	38.6 ± 7.8	52.9 ± 8.5*	35.5 ± 9.9
1000 ng	70.0 ± 3.6***	70.7 ± 3.4***	58.7 ± 3.9**	36.1 ± 13.3	63.0 ± 5.5*	54.8 ± 4.1*
Saline	9.0 ± 1.5	9.6 ± 1.9	9.2 ± 1.8	3.2 ± 0.6	7.1 ± 0.8	10.3 ± 2.2
REM						
100 ng	9.2 ± 1.7	11.6 ± 1.8	9.8 ± 2.4	12.5 ± 9.2	10.5 ± 3.8	10.6 ± 4.0
1000 ng	9.6 ± 1.5	12.6 ± 1.6	10.9 ± 2.0	1.6 ± 1.4	11.3 ± 4.6	7.4 ± 2.5

†The vigilance state durations, expressed in minutes, are analysed per 2-h period for the first 6 h following i.c.v. administration of CST-14 in the light (normal cycle, $n = 12$) or dark period (reverse cycle, $n = 5$). Values are mean ± SEM and asterisks indicate differences from baseline (vehicle injection; Fisher's *posthoc* test following ANOVA *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The SWS2 promoting effect of CST-14 peaks during the first 4-h postinjection periods in normal and reverse cycle, respectively, and decreases during the following 2 h (4–6 h). This effect is observed at the expense of W, and also SWS1 in normal cycle

as observed after saline infusion (Fig. 2A). EEG power spectra profiles in W, SWS1 and REM sleep observed after CST-14 were also indistinguishable from saline. In particular no significant changes of delta power were observed in W, SWS1 and REM sleep (Table 2).

Higher preprocortistatin mRNA concentration with sleep deprivation

Many endogenous substances related to sleep are regulated by the photoperiod and the internal circadian clock (Borbely & Tobler, 1996). To test whether preprocortistatin mRNA expression is regulated during the light : dark cycle, we analysed preprocortistatin mRNA concentrations at different times across a 24-h period by Northern blot. Preprocortistatin mRNA concentrations were significantly lower at the end of the light period and higher during the dark period, when the animals are most active (Fig. 3; $F_{5,15} = 3.614$; $P < 0.05$).

If CST-14 is an endogenous regulator of sleep, insults to the sleep homeostasis should alter its steady-state concentration. To test this hypothesis, we measured preprocortistatin mRNA concentrations in the cortices of six groups of rats. The first group consisted of control animals; a second group had been deprived of total sleep for 24 h by gentle handling; another group of rats had been sleep-deprived by gentle handling and allowed to recover for eight hours; a fourth group was deprived of sleep and let recover for four hours; the fifth group had been deprived only of REM sleep by the platform method for 8 h; and the sixth group served as control for REM deprivation and consisted of rats that were put on a large platform over water. Preprocortistatin mRNA concentration was significantly higher in rats that had been sleep deprived ($F_{2,8} = 8.753$; $P < 0.05$; Fig. 4). Preprocortistatin mRNA levels returned to levels observed in control animals after eight hours of sleep recovery but 4 h of sleep rebound were not sufficient to reestablish control levels of preprocortistatin mRNA (not shown). Pairwise multiple comparisons (Fisher's LSD method) showed significant changes between control and sleep deprived rats ($P < 0.05$), and between sleep deprived and recovered animals ($P < 0.05$), but not between control and recovered animals ($P = 0.422$). In contrast, REM deprivation did not affect preprocortistatin mRNA concentration, when compared with rats in the large platform $F = 0.417$ $P > 0.05$).

The immediate early gene *c-fos* has been extensively used as a marker of neuronal activity (Sagar *et al*., 1988) and its expression has been shown to increase upon sleep deprivation in the cerebral cortex (Grassi-Zucconi *et al*., 1993; Cirelli *et al*., 1996; Basheer *et al*., 1997). To determine the steady-state mRNA concentration of *c-fos* in the samples described in Fig. 4 of sleep-deprived and control rats, we hybridized the same Northern blot with a cDNA probe encoding the transcription factor *c-fos*. Consistent with previous reports, *c-fos* mRNA concentration was increased in the neocortex of sleep deprived animals. Animals that were allowed to recover for eight hours after sleep deprivation showed a dramatic decrease in *c-fos* mRNA concentration, which reached undetectable levels (Fig. 4).

Preprocortistatin partially colocalizes with c-FOS in sleep-deprived rats

To test whether higher preprocortistatin mRNA expression levels upon sleep deprivation were observed in a particular set of cells or in all preprocortistatin-expressing cells we conducted *in situ* hybridization on brain sections from rats that had been deprived of total sleep for 24 h. An overall increase in preprocortistatin mRNA signal per cell was observed throughout all cortical layers (32.3 ± 9.2 silver grains/cell in control animals vs. 58.1 ± 10.3 grains/cell in sleep deprived; 75 cells/animal, $P < 0.05$; Fig. 4B and C). The number of preprocortistatin-positive cells was significantly higher in sleep deprived animals (Fig. 4B and C) throughout the cortex ($P < 0.001$, two-way ANOVA, Fisher's LSD method) with also significant differences according to cortical areas ($P < 0.01$). For instance, in occipital cortex 9.3 ± 2 preprocortistatin mRNA-positive cells/field were observed in sleep deprived rats vs. 15.3 ± 3.2 cells/field found in

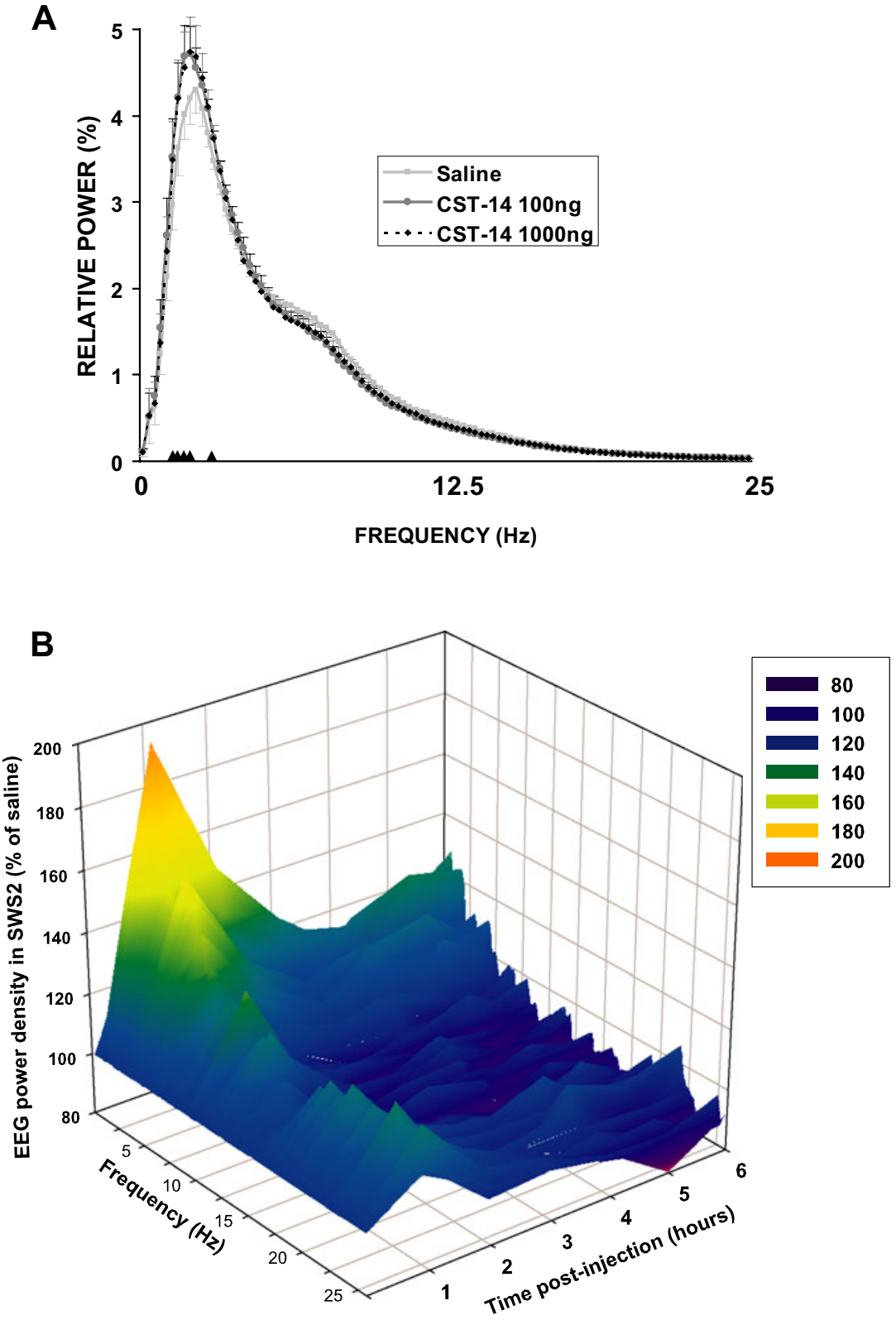

FIG. 2. (A) Spectral profiles for the 6 h of recording following i.c.v. injection of saline (vehicle), 100 ng and 1 μg of CST-14. For each condition, values (mean ± SEM) for each frequency bins (0.25 Hz) are expressed as per cent of the total EEG power (0–25 Hz) during 6 h following the injection. Note that CST-14 administration induces changes in EEG power only in frequency bins from the delta band (repeated measures ANOVA, *posthoc* Fisher's LSD method, CST-14 100 ng vs. saline $P < 0.05$; CST-14 1000 ng vs. saline: $P < 0.05$). (B) Kinetics of EEG power densities after i.c.v. injection CST-14 100 ng. EEG power densities are expressed in percentage of variation from saline condition. The graph represents the values (mean variation per hour) for each frequency bin (0.25 Hz) between 0 and 25 Hz for 6 h of recording. Note that the main change after administration of CST-14 100 ng concerns the low frequency band (delta band) and for the first hours progressively declining over time.

TABLE 2. Time course of slow-wave activity (% change from saline) occurring in the different sleep stages after CST-14 administration

	0–2 h	2–4 h	4–6 h
W	117.1 ± 7.7	103.7 ± 7.1	101.7 ± 9.4
SWS 1	108.0 ± 13.4	93.5 ± 7.4	90.3 ± 10.7
SWS 2	131.3 ± 13.3*	109.4 ± 4.3	108.1 ± 8.9
REM sleep	110.2 ± 13.4	109.7 ± 14.2	103.0 ± 10.1

Values for SWA following CST-14 injection are expressed as percentage of the individual mean level of SWA over the same postinjection period of saline (100%) within the same vigilance states (means ± SEM, 2-h-period values). Raw data have been used for the statistical analysis. Note that the effect of CST-14 on SWA peaks for the first 2 h following infusion of the peptide. The effect of CST-14 on SWA appears to be an on/off effect as there was no difference between the two doses of CST-14 and data from the two doses overlapped. Therefore, we pooled the data from the two doses and used a paired *t*-test to assess the statistical differences.

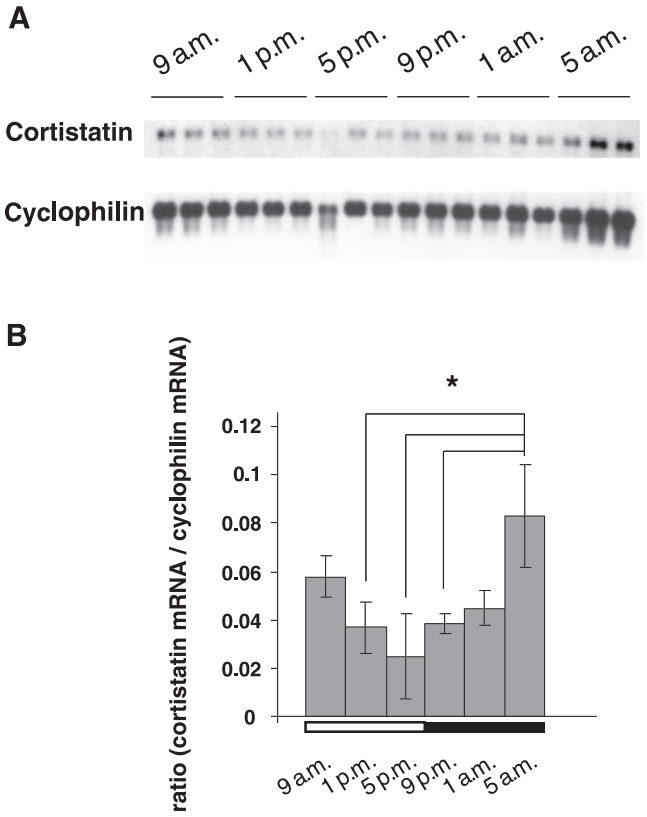

FIG. 3. Preprocortistatin mRNA concentration changes along the light : dark cycle. (A) Northern blot of mRNA samples ($n = 3$) obtained from individual cortices dissected from rats killed at different circadian times. (B) The ratio of the signal intensity between preprocortistatin and cyclophilin is lowest at the end of the light period, when animals have nearly completed their rest period, and is steadily higher during the active (dark) period. *$P < 0.05$ (Single factor ANOVA).

control animals, whereas in somatosensory cortex 45.2 ± 4.2 cells/-field in control animals vs. 79.3 ± 6.6 in sleep deprived rats; ($P < 0.05$). Expression of preprocortistatin mRNA in the hippocampal formation was unaffected.

To determine whether the cell population that increases the concentration of c-FOS during sleep deprivation correlated with the same population of cells that express preprocortistatin mRNA, we used combined immunohistochemistry to c-FOS and *in situ* hybridization to

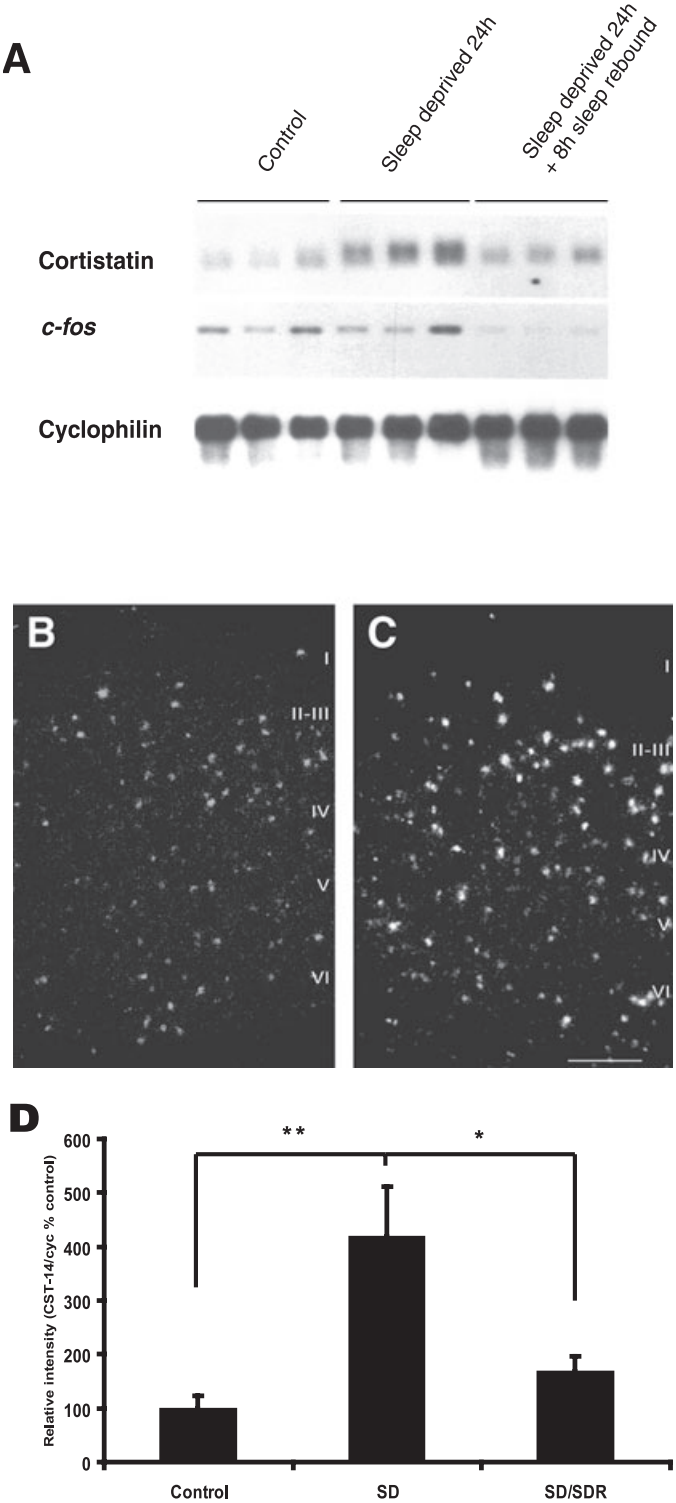

FIG. 4. Preprocortistatin mRNA is higher upon 24-h sleep deprivation. (A) Northern blot of cytoplasmic poly A+ mRNA (2 μg/lane) from cortex of control rats, and rats that had been sleep deprived for 24 h. Preprocortistatin steady-state mRNA concentration are four-fold higher in sleep-deprived rats as compared with controls. Preprocortistatin mRNA concentration returned to control levels after eight hours of sleep rebound. Dark field micrographs of control (B) and sleep deprived rat brain sections (C) hybridized with a full-length riboprobe to rat preprocortistatin mRNA (accession number NM_012835). Note that the signal per cell is significantly higher in sleep-deprived rats. (D) Graph showing the relative intensity of preprocortistatin mRNA in the different sleep deprivation conditions. Values were normalized against cyclophilin signal. **$P < 0.01$; *$P < 0.05$.

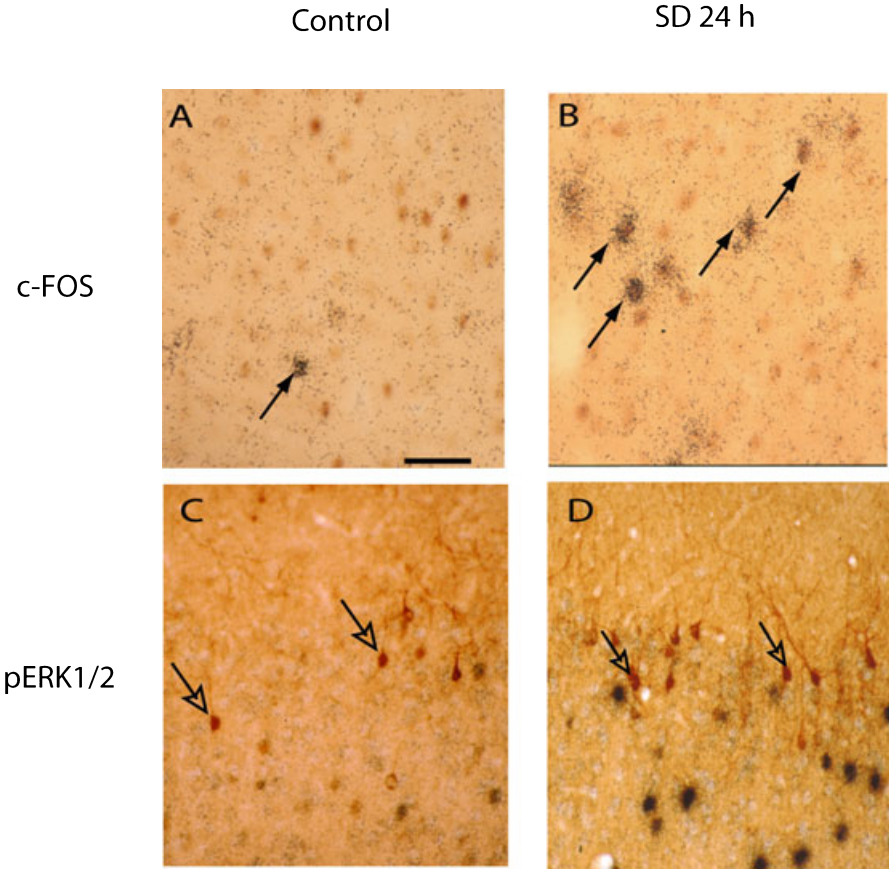

FIG. 5. Colocalization of preprocortistatin mRNA and markers of neuronal activity in control (A and C) and sleep-deprived rats (B and D). Very few preprocortistatin mRNA positive cells showed c-FOS immunoreactivity under control conditions (A). In contrast, the number of c-FOS-CST mRNA double-labelled cells is dramatically higher after total sleep deprivation (B, shown at higher magnification to visualize double-labelled cells). Phosphorylation of ERK1/2 kinases has been associated with cellular stress and synaptic plasticity. The number of preprocortistatin mRNA-positive cells colocalizing with phosphoERK1/2 decreased in the parietal neocortex of sleep deprived rats (D, 2.6 ± 0.5%; Mann–Whitney *t*-test; $P < 0.001$) in comparison with control animals (B, 13 ± 1.4%), suggesting that the induction of *c-fos* is not the direct result of neuronal stress caused by forced waking. Arrows indicate double-labelled cells. Open arrows indicate pERK positive cells. Scale bar, 100 μm.

preprocortistatin in rats that had been deprived of sleep for 24 h. We counted the number of CST-positive, c-FOS-positive and double-positive neurons across the cortical layers in three different cortical areas (motor, somatosensory, and occipital) in control ($n = 464$ cells from 3 different animals) and sleep deprived animals ($n = 505$ cells; three animals). The number of preprocortistatin mRNA/c-FOS-double-positive cells was significantly higher in sleep-deprived rats (3.47 ± 1.9 positive cells/mm^2 vs. 0.37 ± 0.28 cells/mm^2 in control rats; data pooled form all cortical areas; $F_{11,1} = 17.79$ $P = 0.001$, ANOVA). Preprocortistatin mRNA and c-FOS-like immunoreactivity colocalized in neurons in the upper layers of the visual/occipital and somatosensory cortices but not in motor cortex of sleep-deprived animals (Fig. 5A and B). These results suggest that sleep deprivation is associated with changes in the activity of particular sets of neurons, which include preprocortistatin-expressing cells.

In neurons, ERK/MAPK has been shown to be potently activated by phosphorylation after synaptically driven increases in intracellular Ca^{2+} (Impey *et al.*, 1999) and has been associated with plasticity processes in the hippocampus and amygdala (Schafe *et al.*, 2000), as well as with cellular stress (Sabio *et al.*, 2004). To determine the specificity of the molecular changes of cortistatin-expressing cells induced by sleep deprivation, we performed immunohistochemistry to phospho-p44/42 MAPK (pErk1/2) combined with *in situ* hybridization to preprocortistatin mRNA. (Fig. 5C and D). In control conditions, 13 ± 1.4% of cortistatin-expressing cells in the neocortex colocalized with pERK1/2 ($n = 1376$ cells from three animals), whereas in sleep deprived animals this proportion was reduced to 3.6 ± 0.9% ($n = 787$ cells; three animals; Mann–Whitney *t*-test; $P < 0.001$). This result suggests that the increase in cortistatin positive cells is not the result of nonspecific cellular stress caused by sleep deprivation.

Discussion

Cortistatin-14 promotes deep slow-wave sleep and EEG synchronization

Here, we report that CST-14 is hypnogenic even when the peptide was injected at the beginning of the dark period, when the animals have less physiological pressure for sleep. The SWS2 promoting effect could be accounted for by an increase in the duration of the SWS2 episodes without increase in sleep fragmentation, suggesting that CST-14 is primarily involved in maintenance, rather than triggering of EEG synchronization. The increase in SWA within SWS2 observed after CST-14 administration also suggests that the peptide increases the depth of NREM sleep as observed with sleep need. Besides the observed increase of delta power in SWS2, the EEG spectral power spectrum in CST-14-treated animals was undistinguishable from NREM sleep in control animals. The spectra in the other vigilance states were not affected, indicating that the effect of CST-14 was specific to deep SWS.

SWA may also be a sign of synaptic plasticity occurring during sleep (Steriade & Timofeev, 2003) and has been proposed to be associated with synaptic downscaling (Tononi & Cirelli, 2003). Monocular deprivation experiments have shown that sleep enhances synaptic remodeling during the critical period of visual cortex development (Frank *et al.*, 2001). CST-14 might be involved in the synaptic plasticity associated with synaptic rewiring after monocular deprivation, as its expression correlates with sleep and is elevated during visual cortex development (de Lecea *et al.*, 1997). A role for CST-14 in synaptic plasticity has recently been proposed, as transgenic mice overexpressing CST-14 show alterations in hippocampal LTP and learning (Tallent *et al.*, 2005), due to a direct inhibitory effect in hippocampal neurons. Cortical activity in these

mice, however, was not affected significantly. Altogether, these data suggest that CST-14 may play a role in learning/synaptic plasticity related to slow-wave activity during sleep.

Cortistatin gene expression negatively correlates with NREM sleep

We have shown that the mRNA coding for the endogenous neuropeptide CST-14 in the cerebral cortex is higher upon sleep deprivation, and its mRNA also accumulates during the active dark period. Conversely, preprocortistatin steady-state mRNA concentration decreases progressively with time spent in NREM sleep while it shows minimum levels at the end of the rest (light) period. We can hypothesize that NREM sleep decreases the turnover of preprocortistatin mRNA synthesis, as we cannot rule out that its steady-state levels accumulate upon physiological demand. However, the preprocortistatin mRNA steady-state levels are four-fold higher under sleep deprivation as compared with controls, which were harvested at light onset, the circadian peak of preprocortistatin expression. Therefore, preprocortistatin mRNA levels observed after sleep deprivation are higher than those found during any other circadian fluctuation, suggesting that preprocortistatin transcription cannot decrease in the absence of sleep. Our results from REM deprivation might suggest that increasing wake time had no effect on preprocortistatin mRNA expression. Also, the REM deprivation data indicate that the increased effects during forced waking are not a direct product of stress. Moreover, this latter observation demonstrates that the decrease of CST transcription with sleep is not correlated with REM sleep, although it is with NREM sleep.

Sleep deprivation increases the turnover and mRNA synthesis of several molecules associated with cellular stress (Cirelli, 2005). Sleep recovery after a 24-h total sleep deprivation is almost complete after 8 h of rebound (Franken *et al*., 1991; Franken, 2002), which seems to correlate with the kinetics of decrease of preprocortistatin expression to return to control values. Preprocortistatin mRNA expression is also modulated across the 24-h light : dark cycle, especially at the transition between lights off and on. Although preprocortisatin is not expressed in areas directly regulated by light, we cannot rule out that release of CST-14 is affected indirectly by light-sensitive subcortical structures. Finally, while these results will have to be confirmed at the protein level when selective antibodies to CST-14 become available, our data suggest that preprocortistatin mRNA expression inversely correlates with time spent in SWS.

Preprocortistatin mRNA is induced upon sleep deprivation in cortical neurons coexpressing c-FOS

Preprocortistatin mRNA and c-FOS immunoreactivity are colocalized in a subset of cortical neurons of sleep-deprived rats. Even considering the technical limitations of *c-fos* mapping (mono- vs. polysynaptic activity, difference in signal transduction pathways, etc.) our results can be interpreted as higher activation of CST-14-producing neurons during wakefulness than during sleep. Part of the response could be accounted for by stress, inherent to sleep deprivation. However, c-FOS staining was selectively enhanced in the neocortex, and was very low in areas associated to stress, such as the amygdala or hypothalamic structures.

The transition from sleep to wakefulness is accompanied in the cortex by increases in immunoreactivity to *c-fos*, and other immediate-early genes (Cirelli & Tononi, 1998, 2000), that have been associated with neuronal activation. These results raise the question whether *c-fos* expressing neurons represent particular populations of cells. Our double labelling experiments suggest that most c-FOS-positive cells are pyramidal cells. However, a significant number of preprocortistatin mRNA positive cells colocalize with c-FOS immunoreactivity in the upper layers of the neocortex upon sleep deprivation. As cortistatin is exclusively expressed in GABAergic neurons in the cerebral cortex (de Lecea *et al*., 1997), the c-FOS data suggest that a subset of interneurons is activated during prolonged wakefulness. We also show phosphoERK1/2 levels, another signal transducer involved in plastic phenomena (Impey *et al*., 1999), are reduced in preprocortistatin-positive cells upon sleep deprivation. Together, these results suggest that specific subtypes of neurons and signal transduction pathways have a lower level of activation in local cortical regions during sleep in comparison with sleep, and may be reflected in local variations in the EEG across process S (Vyazovskiy *et al*., 2002). CST-14 expressing neurons may be important and active during the recovery period to promote maintenance of cortical synchrony. The relative role of these interneurons on the control of cortical synchronization remains to be established.

How does CST-14 promote sleep?

The enhancement of the mean duration of SWS2 episodes by CST-14 suggests that the peptide plays a role in the sleep/wakefulness transitions by maintaining cortical synchronization and/or inhibiting desynchronization. CST-14 may synchronize cortical synchrony through multiple mechanisms including direct inhibition of pyramidal neurons (de Lecea *et al*., 1996) and enhancement of thalamocortical rhythmicity by enhancing Ih (Schweitzer *et al*., 2003) This cationic conductance has been shown to be responsible for the generation of thalamocortical oscillations (McCormick & Bal, 1997), and may also be involved in the maintenance of intrinsic cortical rhythms (Mao *et al*., 2001). Third, as anticipated in our previous studies (de Lecea *et al*., 1996), it is possible that CST-14 promotes sleep by antagonizing the effects of acetylcholine in cortical excitability. Subcortical cholinergic afferents are known to block the network- and intrinsically generated slow-wave oscillations, thus promoting wakefulness activity patterns in cortical and thalamic neurons (Steriade *et al*., 1993). It has been suggested that high levels of ACh, as observed during both brain activated states of W and REM sleep (Steriade, 2004) may set suitable conditions for encoding new information in the hippocampus. Also, low acetylcholine levels during sleep appear to be critical for declarative memory consolidation (Gais & Born, 2004).

Together, our data suggest that preprocortistatin mRNA levels inversely correlate with time spent in SWS and is released during sleep. Accumulation of CST-14 above a certain threshold would promote and maintain SWA that characterizes sleep need.

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Abbreviations

CST-14, cortistatin; EEG, electroencephalogram; EMG, electromyogram; LSD, least significant difference; NREM, nonrapid-eye-movement; REM, rapid-eye-movement; SRIF-14, somatostatin; SWA, slow-wave activity; SWS, slow-wave sleep; W, wakefulness.

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